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Action Potentials Occur Spontaneously in Squid Giant Axons with Moderately Alkaline Intracellular pH

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Abstract. This report demonstrates a novel finding from the classic giant axon preparation of the squid. Namely, the axon can be made to fire autonomously (spontaneously occurring action potentials) when the intracellular pH (pH_i) was increased to about 7.7, or higher. (Physiological pH_i is 7.3.) The frequency of firing was 33 Hz ($T = 5°$). No changes in frequency or in the voltage waveform itself were observed when pH_i was increased from 7.7 up to 8.5. In other words, the effect has a threshold at a pH_i of about 7.7. A mathematical model that is sufficient to mimic these results is provided using a modified version of the Clay (1998) description of the axonal ionic currents.

Introduction

The electrical response of squid giant axons *in vivo* to environmental stimuli can be characterized as being primarily phasic. For example, the axon fires an action potential once, and only once, in 15 °C seawater in response to a light flash, thereby triggering the rapid, jet-propelled escape of the animal (Otis and Gilly, 1990). A more complicated behavior of the axon occurs in concert with the parallel small axon system (Young, 1939) during the delayed jet escape from chemical stimuli applied at the olfactory organ (Otis and Gilly, 1990). Under these conditions the giant axon fires from one to three action potentials (or none at all). A reduction in temperature to 6 °C, which squid often encounter in deep waters, produces changes in the role of the axon in these behaviors (Neumeister *et al.*, 2000). For example, the axon usually fires twice in response to a light flash at 6 °C (Neumeister *et al.*, 2000). A tonic train of a relatively large number of action potentials does not appear to be elicited *in vivo*. These results are mirrored by the response of the axon *in vitro* to current stimuli applied with the standard axial wire recording technique. Under these conditions one, and only one, action potential is elicited with a rectangular current pulse, regardless of pulse duration or pulse amplitude (Clay, 1998). Moreover, an action potential is not elicited with a relatively slow depolarizing current ramp (J. R. Clay, unpub. obs.). A rapidly changing stimulus, such as a current step of sufficiently large amplitude, is required.

Given the above results, we were surprised to observe tonic firing of the axon when the pH of the perfusate used during recordings from intracellularly perfused axons was increased to 7.7, or higher. The normal intracellular pH (pH_i) is 7.3 (Boron and DeWeer, 1976). Under these conditions of slightly elevated pH_i, action potentials occurred spontaneously and repetitively. The activity lasted for as long as a few hours in some preparations. An ionic mechanism underlying this observation is proposed.

Materials and Methods

Experiments were performed on internally perfused squid giant axons at the Marine Biological Laboratory, Woods Hole, Massachusetts, using standard axial wire voltage- and current-clamp techniques described elsewhere (Clay and Shlesinger, 1983; Clay, 1998). The intracellular perfusate consisted of 300 m*M* K glutamate and 400 m*M* sucrose, with the pH adjusted to the desired level within the 7.2 to 8.5 range by free glutamic acid. In a few experiments the intracellular buffer consisted of 400 m*M* sucrose, 250 m*M* KF, and 25 m*M* K_2HPO_4 ($pH_i = 7.6–7.8$). The extracellular solution was either filtered seawater (pH = 7.5) or artificial seawater consisting of 430 m*M* NaCl, 10 m*M* KCl, 50 m*M*

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$MgCl_2$, 10 m*M* $CaCl_2$, and 10 m*M* Tris-HCl (pH 7.2). These extracellular solutions were used interchangeably given that similar results were obtained in either condition. The temperature was in the 4–6 °C range; in any single experiment it was maintained constant to within 0.1 °C by a Peltier device located within the experimental chamber. Input resistance measurements were made with rectangular current pulses applied to axons in extracellular medium containing tetrodotoxin (TTX, Sigma Chemical Co.) at a final concentration of 1 μM.

Computer simulations of membrane excitability were carried out as described previously (Clay, 1998). The model is given by

$$C dV/dt + (I_K + I_{Na} + I_{NaP} + I_L + I_{K,ir} + I_{stim}) = 0,$$

where V is membrane potential in mV, t is time in ms, C is the specific membrane capacitance ($C = 1\ \mu F \cdot cm^{-2}$), I_{stim} is the stimulus current ($\mu A \cdot cm^{-2}$), and the various ionic current components are described as follows. The sodium ion current is given as in Vandenberg and Bezanilla (1991) with

$$I_{Na} = g_{Na} P_O V(\exp((V - E_{Na})/24) - 1)/((\exp(V/24) - 1) \times (1 + 0.4 \exp(-0.38V/24)))$$

where $g_{Na} = 107\ mS \cdot cm^{-2}$, $E_{Na} = 64$ mV, and P_O is the probability that any single Na^+ channel is in the open state of the Vandenberg and Bezanilla (1991) kinetic scheme. The various rate constants in the model (in ms^{-1}) are as follows:

$a = 7.55 \exp(0.017(V - 10))$,
$b = 5.6 \exp(-0.00017(V - 10))$,
$c = 21.0 \exp(0.06(V - 10))$,
$d = 1.8 \exp(-0.02(V - 10))$,
$f = 0.56 \exp(0.00004(V - 10))$,
$g = \exp(0.00004(V - 10))$,
$i = 0.0052 \exp(-0.038(V - 10))$,
$j = 0.009 \exp(-0.038(V - 10))$,
$y = 22.0 \exp(0.014(V - 10))$,
$z = 1.26 \exp(-0.048(V - 10))$.

The potassium ion current is given by

$$I_K = g_K n(V, t)^8 V(\exp(V/24) - K_s(t)/K_i)/(\exp(V/24) - 1),$$

where $g_K = 62.5\ mS \cdot cm^{-2}$, $dn(V, t)/dt = -(\alpha + \beta)n(V, t) - \alpha$, with $\alpha = -0.0075(V + 64)/(\exp(-0.11(V + 64)) - 1)$ and $\beta = 0.075 \exp(-(V + 62)/20)$. This represents a modification of the model of I_K in Clay (1998) in which n^4 kinetics were used. The α and β parameters have also been modified so as to obtain equivalent (or better) descriptions of the I_K results given by the n^4 model in Clay (1998). The K_s parameter, which corresponds to the potassium ion concentration in the restricted space just outside of the axolemma, is given by

$$dK_s/dt = 0.0104 I_K - 0.08(K_s - 10) - 5(K_s - 10)/(1 + (K_s - 10)/2)^3.$$

Further details concerning the description of the I_{Na} and I_K components are given in Clay (1998). The background, or time-independent current in the model consists of three terms: the "leak" current, I_L; a persistent, tetrodotoxin-sensitive sodium ion current I_{NaP} (Rakowski *et al.*, 1985); and an inwardly rectifying potassium ion current, $I_{K,ir}$. The latter, together with I_{NaP}, confers nonlinearity to the background current in the −90 to −60 mV range, similar to that observed experimentally. These components are described by

$$I_{NaP} = 4.5(V/24)(0.03 \exp(V/24) - 0.43)/((\exp(V/24) - 1) \times (1 + \exp(-(V + 65)/7)));$$

$$I_{K,ir} = 0.24(V + 82)/(1 + 0.05(\exp(0.15(V + 82)))); \quad \text{and}$$

$$I_L = g_L(V + 49),$$

where g_L is either 0.2 ($pH_i = 7.3$) or 0.03 (pH_i 8.5). The formulation for $I_{Na,P}$ represents a best fit, by eye, to unpublished measurements of this component kindly provided by R. F. Rakowski (Finch University of Health Sciences/The Chicago Medical School).

The simulations were implemented with a fourth-order Runga-Kutta iteration routine in FORTRAN with a time step of 1 μs.

Results

As noted above, the physiological intracellular pH for squid giant axons is 7.3 ± 0.013 (±SE; Boron and DeWeer, 1976). Under these conditions axons are quiescent with a resting potential of about −60 mV. An action potential elicited by a brief current pulse is shown in Figure 1A. A slight oscillatory rebound following the action potential was apparent, as indicated by the arrow in Figure 1A. The effect of changing the intracellular pH to 8.5 is illustrated in Figure 1B and C. A few minutes after the solution change, the membrane potential became oscillatory (inset, Fig. 1B). Moreover, it exhibited a much greater post-excitatory rebound (Fig. 1B). Several minutes later, the amplitude of the spontaneously occurring subthreshold oscillations increased, followed by a train of action potentials that lasted until the experiment was terminated. The result in Figure 1C occurred shortly after the change in pH_i. Similar recordings were obtained at later times in these experiments by clamping the membrane potential at the equilibrium point and then slowly releasing the clamp. The membrane potential remained at the equilibrium point for a few seconds and then began to oscillate spontaneously. The oscillations increased in amplitude until an unending train of action potentials occurred, similar to the result in Figure 1C. Results

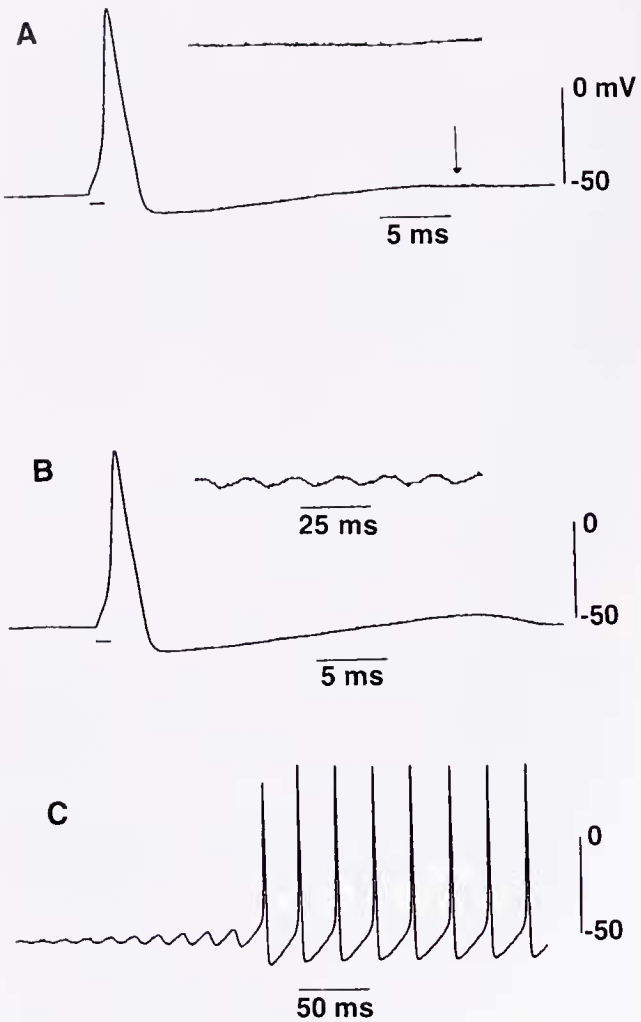

Figure 1. (A) Action potential from a squid giant axon in control conditions (pH_i = 7.3) elicited by a 1-ms, suprathreshold current pulse. The arrow indicates a slight oscillatory rebound after the action potential. The inset illustrates a 100-ms epoch during rest (voltage scale 4×). (B) Action potential 2 min after changing to an intracellular buffer with pH = 8.5. The oscillatory rebound was considerably larger, and the membrane potential oscillated about the resting level (inset; voltage scale 4×). (C) Initiation of spontaneous firing of action potentials, about 3 min after the change to pH_i = 8.5. The spontaneous activity in this preparation lasted 4 h, at which point the experiment was terminated.

such as those in Figure 1 were observed in 20 out of 24 axons in which the effect was investigated. The frequency of firing at T = 5 °C, the temperature at which several of the experiments were performed, was 32.9 ± 6.1 Hz (n = 8; ±SD).

The pH_i effect was reversible, as illustrated in Figure 2. In this experiment, pH_i was initially 8.3. The axon fired spontaneously (Fig. 2A). The intracellular perfusate was then switched to one having a pH of 7.7 (Fig. 2B). No clear effect on the electrical activity was observed 15 min after the solution change. When the intracellular buffer was changed to one having pH = 7.4, the activity ceased, although small-amplitude subthreshold oscillations were still observed (Fig. 2C). Spontaneous activity was reestablished when the initial perfusate (pH_i = 8.3) was used (Fig. 2D). These results are consistent with a threshold (all-or-none phenomenon) for autonomous activity with pH_i. The threshold was in the 7.6 to 7.8 range, as indicated by four experiments in which pH_i was changed in 0.2 increments from pH_i = 7.2 to 8.4.

In three experiments on an unrelated topic, spontaneous firing was observed upon initiation of intracellular perfusion with a buffer consisting of 400 m*M* sucrose, 250 m*M* KF,

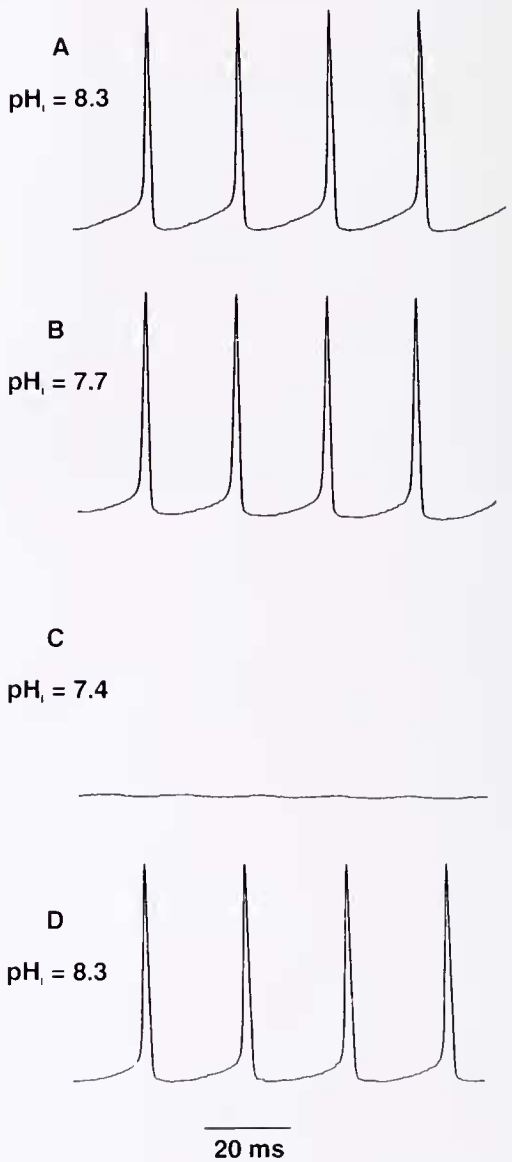

Figure 2. Autonomous activity with pH_i = 8.3 (A) and pH_i = 7.7 (B). No clear effect was apparent with this change in pH. Spontaneous activity ceased with pH_i = 7.4 (C), although subthreshold oscillations were apparent. (D) Spontaneous activity was re-established with pH_i = 8.3. Different preparation than in Figure 1.

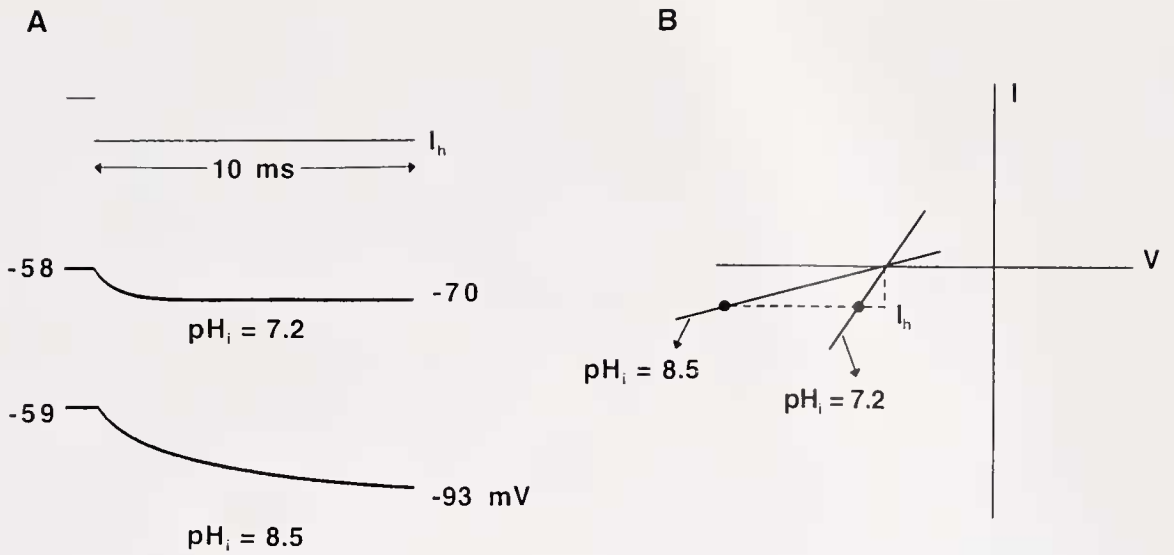

Figure 3. Effect of pH_i on background ("leak") conductance. (A) Membrane potential responses to a 30 $\mu A \cdot cm^{-2}$ hyperpolarizing current pulse with pH_i = 7.2 and 8.5, as described in the text. (B) Schematic description of the change in the current-voltage relation of the axon which is proposed to explain the results in panel A.

and 25 m*M* K_2HPO_4 (pH_i = 7.6–7.8). These results demonstrate that the effect was not a function of the buffering system. We primarily used K glutamate which, as Wanke *et al.* (1980a) noted, is appropriate at a concentration of 45 m*M* for pH in the 9 to 10.8 range. We found that a solution containing 300 m*M* K glutamate could be stably titrated (with free glutamic acid) down to pH 7.2, which allowed us to cover the pH range of interest (7.2 to 8.5) with a single buffer system containing an anion—glutamate—which is known to be "favorable" for squid giant axons (Adams and Oxford, 1983; Clay, 1988).

The most logical place, *a priori,* to look for the ionic mechanism underlying the pH_i effect would seem to be the classical sodium and potassium ion currents, I_{Na} and I_K, respectively, that underlie the action potential (Hodgkin and Huxley, 1952). We looked for an effect of a change in pH_i on I_{Na} in voltage-clamp recordings with pH_i in the 7 to 9 range, but we did not observe any clear effect. An irreversible reduction of I_{Na} inactivation does occur for a pH_i greater than 9.5 (Brodwick and Eaton, 1978), which is outside the range of pH_i we have used. Moreover, blockade of I_{Na} in squid axons by intracellular protons has been observed having pK_a values of 4.6 and 5.8 (Wanke *et al.*, 1980b)—an effect of pH_i which, again, lies outside the range we have used. We are not aware of any report in the literature of an effect of a change in pH_i in the 7 to 9 range on I_{Na}. No such effect was observed in this study.

An increase of pH_i in the 7 to 9 range increases the amplitude of I_K at any given depolarization from a holding level of −50 mV (Wanke *et al.*, 1980a), although a similar effect does not occur with relatively negative holding potentials (−80 or −90 mV; Clay, 1990). This holding potential dependence is consistent with a rightward shift of the I_K inactivation curve along the voltage axis as pH_i is increased in the 6 to 10 range (Clay, 1990). The effect—essentially an increase in the number of K^+ channels available for activation during the action potential—cannot account for pH_i-induced automaticity (simulations not shown).

A clue to the ionic mechanism for pH_i-induced automaticity was provided by input resistance measurements in axons made quiescent with tetrodotoxin (TTX; 1 μM—Fig. 3). The preparation illustrated in Figure 3A rested at −58 mV in TTX with pH_i = 7.2. A hyperpolarizing current pulse 10 ms in duration produced a hyperpolarizing response having a time constant, τ, of 0.7 ms. In an equivalent circuit model of the membrane, this result is equal to the product of the membrane resistance and the membrane capacitance. The specific membrane capacitance is 1 $\mu F \cdot cm^{-2}$. Consequently, the specific membrane resistivity with τ = 0.7 ms is 0.7 $k\Omega \cdot cm^{-2}$, a value that is consistent with the classical small-impedance measurements in figure 23 of Hodgkin and Huxley (1952). The corresponding result for pH_i = 8.5 is shown in the bottom panel of Figure 3A. The change of pH_i from 7.2 to 8.5 produced a slight hyperpolarization of rest potential by about 1 mV. The response to a current pulse of the same amplitude as in pH_i = 7.2 produced a marked increase in membrane hyperpolarization with a much slower response time. Indeed, the response was not yet at the steady-state level at the end of the 10-ms pulse. Similar observations were made in four different preparations. This result is consistent with a reduction of net inward current, as illustrated schematically in Figure 3B. A hyperpolarizing current pulse having an amplitude of I_h intersects the current-voltage relation at a much more negative potential with

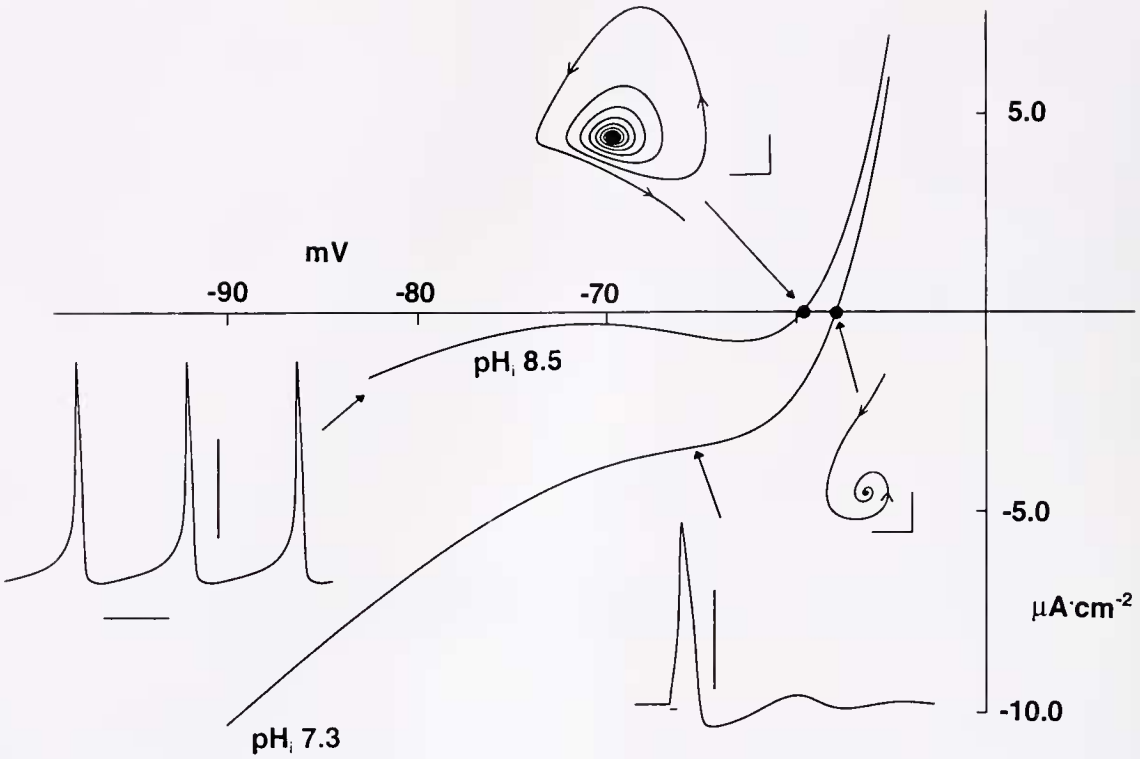

Figure 4. Simulations of pH_i-induced excitability. Steady-state current-voltage relations are shown for pH_i = 7.3 and pH_i = 8.5. The sole difference in the model for the two conditions is in the leak current conductance, which is 0.2 and 0.03 mS · cm^{-2} for pH_i = 7.3 and 8.5, respectively. The equilibrium potential, that is, the point where the current-voltage relation crosses the voltage axis, is stable for pH_i 7.3, as indicated by the trajectory in the inset adjacent to this point. (The scales are 5 mV and 2 μA · cm^{-2}). An action potential elicited by a suprathreshold current pulse for this condition is shown below the current-voltage relation. (The scales are 50 mV and 1 ms.) The equilibrium point for pH_i = 8.5 is unstable, as illustrated by the adjacent trajectory. (Same scales as the current-voltage trajectory for pH_i = 7.3.) This trajectory spiraled out to the limit cycle described by the spontaneous action potentials shown adjacent to the current-voltage relation. Scales are 50 mV and 20 ms.

pH_i 8.5 as compared to pH_i 7.2 (symbols (●) in Fig. 3B). This result suggests that a change of pH_i might affect the third component of the Hodgkin and Huxley (1952) model of the action potential, namely the background, or "leak" current, I_L. In particular, the resistance measurements in Figure 3 imply that the leak component is reduced by an increase in pH_i. This idea has precedence in the work of Bevan and Yeats (1991), who reported the activation of a sustained, nonspecific cation conductance in a subpopulation of rat dorsal root ganglion neurons by extracellular protons. Alkaline pH would reduce the amplitude of this conductance.

The background current in squid giant axons also consists of a small-amplitude tetrodotoxin-sensitive sodium ion current (referred to as I_{NaP}) that is activated at relatively negative potentials, about −80 mV, and has a peak amplitude at −60 mV (Rakowski *et al.*, 1985). This component has a current-voltage relation with a negative slope character at subthreshold potentials, whereas I_L—a net inward current component at subthreshold potentials—has an approximately linear current-voltage relation. We are proposing that the reduction of I_L with increasing pH_i allows the negative slope character of I_{NaP} to destabilize the equilibrium point (rest potential) of the axon at −60 mV, thereby resulting in autonomous activity. We cannot exclude a pH_i dependence of I_{NaP}. However, the pH_i-induced change in resistance illustrated in Figure 3 is not attributable to changes in I_{NaP}, since those experiments were carried out in the presence of TTX, and as shown below, this resistance change is sufficient to explain the pH_i-induced automaticity.

The mechanism we propose for the result in Figure 3 is illustrated by the simulations and current-voltage relations in Figure 4. These results are based on the equations provided in the Materials and Methods. The steady-state current voltage relation of the model for control conditions (pH_i 7.3) for $-90 < V < -55$ mV is shown in Figure 3, along with an action potential (AP) elicited by a brief, suprathreshold current pulse. Only a single AP was elicited in the model even by relatively long-duration current pulses—regardless of pulse amplitude—as in the earlier analysis

(Clay, 1998). The stability of the model for pH_i 7.3 is illustrated by the current-voltage trajectory in the inset of Figure 3 immediately below the pH_i 7.3 equilibrium point. In this simulation the membrane potential was abruptly shifted a few millivolts away from equilibrium conditions. The current-voltage trajectory subsequently spiraled toward the resting potential (stable focus). The effect of changing pH_i to 8.5 is also shown in Figure 3. The *sole* change in the model was a reduction of the leak current conductance, g_L, from 0.2 to 0.03 mS · cm^{-2}. This change resulted in a hyperpolarization of the equilibrium point from −57.6 to −59.3 mV and a change in its stability properties from a stable to an unstable focus (inset above the voltage axis in Fig. 3). This trajectory spiraled toward a stable limit cycle, that is, autonomous firing, as illustrated by the inset to the left of the current-voltage trajectory, with a frequency of firing of 29.8 Hz.

Discussion

Excitability of the squid giant axon preparation *in vitro* has traditionally been increased by reductions in the extracellular Ca^{2+} concentration (Huxley, 1959; Guttman and Barnhill, 1970). In preliminary experiments, we occasionally observed autonomous activity with ¼ normal Ca^{2+} (2.5 m*M*), but the effect was transient and episodic. In all preparations examined, repetitive firing was not observed either autonomously or with current pulse stimulation 15–20 s after the change to an external medium that was low in Ca^{2+}. Moreover, axons became inexcitable within a few minutes in low Ca^{2+} seawater. This result is not surprising given that low Ca^{2+} external medium is deleterious for neurons (Horn, 1999). The pH_i-induced autonomous activity we report here is reproducible, robust, and long-lasting. In one axon, we observed stable repetitive firing for 4 h (with perfusion both intracellularly and extracellularly to maintain ionic gradients), at which point the experiment was terminated. Consequently, this preparation may be an ideal single-cell neuronal oscillator suitable for investigations concerning mechanisms of rhythmicity.

The ionic model that we propose for the spontaneous activity is novel and counterintuitive, in that the effect is attributable to a *reduction* of inward current, thereby leading to a destabilization of the rest potential by the I_{NaP} component. The result in Figure 2C illustrating subthreshold oscillations that increase in amplitude until the threshold for an action potential is reached is consistent with this aspect of the model. The only stable element both in the preparation and the model is the limit cycle, that is, the trajectory traversed in current-voltage space by the action potential (Winfree, 1980).

Repetitive firing in nerve cells is well known in a number of preparations, such as gastropod neuronal somata (Connor and Stevens, 1971). The rapidly inactivating potassium ion current, I_A, is believed to play a major role in the activity (Connor and Stevens, 1971). The delayed rectifier, I_K, in squid giant axons also inactivates, as originally shown by Ehrenstein and Gilbert (1966). We think that this kinetic feature does not play a role in our observations because the inactivation kinetics are shifted rightward along the voltage axis by an increase in pH_i (Clay, 1990), and the onset of inactivation at 5 °C is too slow to be a factor during the relatively brief times the membrane potential is at depolarized potentials during the action potentials in the pulse train (Clay, 1989). Moreover, g_K inactivation cannot account for the destabilization of the resting potential illustrated in Figure 1C, which we believe is the key feature underlying our results.

To our knowledge, the effect, reported here, of pH_i on excitability in squid axons has not been previously reported. A similar effect with pH_o was noted in passing by Bicher and Ohki (1972) in their work with intracellular pH electrodes. They observed an increase in excitability in the giant axon, including autonomous firing in some preparations, after the pH of the extracellular bathing medium was raised to 9. The change in pH_o caused a few tenths rise in pH_i, which we have shown to be sufficient to induce automaticity. It is tempting to speculate that our observations have physiological relevance, given that they occur within the normal range of pH in the ocean (7.5 to 8.4; Sverdrup *et al.*, 1942), and only slightly above the normal, relatively alkaline, value of 7.3 in the axon (Boron and DeWeer, 1976). Moreover, transient rises in pH in squid blood have been reported in exercising squid (Pörtner *et al.*, 1991), which, based on our work, would favor an increase in neuronal excitability. However, not enough is known about the role of pH_i in squid behavior to make an informed conjecture about the role of the increased excitability *in vivo*, if it indeed occurs.

Acknowledgments

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